

Degradation Characteristics of Isolated and *In Situ* Cell Wall Lucerne Pectic Polysaccharides by Mixed Ruminal Microbes*

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Abstract: Two pectic polysaccharide fractions were isolated from lucerne (*Medicago sativa* L) leaves and used in fermentation experiments with mixed ruminal microbes. Both fractions were similar in chemical composition, containing galacturonic acid (52–58 mol%) and the neutral sugars arabinose (14–18 mol%), galactose (6–8 mol%) and rhamnose (8–12 mol%). Fermentation of both fractions was rapid and complete with a half-life of approximately 4 h. Production of total volatile fatty acids matched the degradation profile reaching a maximum level shortly after the rate of degradation began to decrease. The fermentation characteristics of citrus pectin and polygalacturonic acid were similar to those of the lucerne pectic fractions but galacturonic acid was much slower in its rate of degradation while soluble arabinogalactan from larchwood was virtually undegraded. Leaves of early bud stage lucerne and lower nodes and internodes of stems from full bloom lucerne were also fermented by mixed ruminal microbes. Pectic polysaccharides were rapidly and extensively degraded from both tissues. Initial rates were faster for leaves than for stems and the extent of pectic degradation was greater in leaves (8% residual) than in stems (17% residual). Selection of forage lines with increased pectic polysaccharides would provide greater amounts of rapidly available energy that could result in more efficient utilisation of the rapidly degraded protein in lucerne.

Key words: lucerne, pectic polysaccharides, cell wall, ruminal microbes, degradation

INTRODUCTION

Lucerne has many advantages over other forage crops utilised by the dairy industry and other ruminant-based enterprises. Among these is the high protein level found in the leaves even at relatively mature stages of development. The full benefit of this high protein content is not realised due to high degradation rates and conversion to ammonia in the rumen, leading to increased nitrogen

excretion. Two strategies are possible to improve utilisation of plant protein. The first is to alter the proteins to decrease their rate of degradation. Schemes to alter proteins range from chemical or physical treatments to genetically changing the plant to produce more resistant proteins or protective compounds (eg tannins) (Broderick *et al* 1991). A second strategy involves matching the rapidly degraded protein with an equally rapidly degraded carbohydrate source such that energy would not be limiting to ruminal microbes (Stokes *et al* 1991a, b). This assures that the protein would be efficiently converted to microbial biomass (including protein) benefiting the ruminant. Pectic polysaccharides represent a potential source of rapidly degradable carbohydrate (Gradel and Dehority 1972). A significant

* Names of manufacturers or suppliers are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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proportion of the total structural polysaccharides in lucerne cell walls is pectin (Hatfield 1992), ranging from 100 to 200 g kg⁻¹ in stems and 250–300 g kg⁻¹ in leaves.

Degradation of pectic polysaccharides by ruminal microbes has been investigated by several groups (Howard 1961; Dehority *et al* 1962; Dehority 1969; Gradel and Dehority 1972; Marounek *et al* 1985; Osborne and Dehority 1989). Previous work characterising pectic polysaccharide degradation has been confined to the utilisation of readily available commercial pectins primarily from the citrus industry. Pectic polysaccharides of lucerne are a heterogeneous group of molecules containing a number of sugars arranged in complex structural arrays (Aspinall and Fanshawe 1961; Aspinall *et al* 1968; Aspinall and Molloy 1968; Hatfield 1991). No studies have been undertaken to determine the degradation patterns and fermentation characteristics of pectic polysaccharides isolated from lucerne walls or any other forage. Typical lucerne pectic polysaccharides contain four major sugar residues (arabinose (Ara); galactose (Gal); rhamnose (Rha); and galacturonic acid (GalA)) combined into complex molecules that may contain up to 20 different linkages/substitution patterns (Hatfield 1991). Commercially available pectic polysaccharides are refined subfractions that often lack this structural complexity. Complete degradation of lucerne pectic polysaccharides requires a large array of wall hydrolases.

Chesson and Monro (1982) investigated the *in situ* degradation of pectic substances from red clover and lucerne forages using a nylon bag technique and a rumen-cannulated sheep. They separated plants into leaf and stem fractions and monitored degradation by the changes in the solubilisation patterns of carbohydrates in water and ammonium oxalate extracts. Based on the rapid decline in carbohydrates solubilised by the two treatments they concluded that pectic polysaccharides were rapidly degraded from walls, with removal being nearly complete in 18 h. Recently, Titgemeyer *et al* (1992) extended this earlier work to include all wall carbohydrates from leaf and stem fractions. The approach was similar utilising a wall fractionation scheme in combination with a total wall hydrolysis to monitor changes in the digestibility of lucerne walls. They also concluded that pectic polysaccharides were rapidly and nearly completely degraded after 12 h. However, recent work has shown that not all of the pectic polysaccharides are easily solubilised from the lucerne wall. A portion of these polysaccharides, particularly in stem tissues, is resistant to extraction until the walls have been delignified (Hatfield 1992). None of the previous studies have considered this wall fraction. Depending upon the plant sample this can account for as much as 300 g kg⁻¹ of the total pectic polysaccharides (Hatfield 1992). Limitations to solubilisation may indicate structural features and/or

interactions that restrict pectic polysaccharide degradation by rumen microflora, rendering this fraction unavailable to the animal.

We were interested in understanding the degradation/fermentation characteristics of isolated lucerne pectic polysaccharides and in comparing these with the degradation pattern of the total pectic fraction contained within lucerne walls. Our aim was to determine the potential benefits from the rapid degradation of pectic polysaccharides and to determine if there would be a benefit from selection of forages with increased pectic polysaccharides in the wall fraction of the biomass. To obtain this information we had four main experimental objectives. (1) Isolate lucerne pectic polysaccharides, determine their degradation/fermentation characteristics by rumen microbes and compare these characteristics with some commercially available polysaccharides. (2) Characterise the metabolic products produced from isolated pectic fractions of lucerne to determine their impact on rumen pH and if there would be an undesirable shift in fermentation endproduct ratios. (3) Compare the degradation characteristics of isolated pectic polysaccharides with the same fractions in intact walls. (4) Determine if the pectic fraction that was resistant to extraction was also resistant to degradation, a critical factor in developing screening protocols.

MATERIALS AND METHODS

Lucerne (*Medicago sativa* L) plants were grown in a greenhouse under high pressure sodium lamps using a 14 : 10 h day : night light regime. Plants were harvested 7.5 cm above the soil line at the appropriate developmental stage, freeze-dried and separated into leaves with petioles attached and stems. For isolation of pectic polysaccharides, plants were harvested at the early bud stage. For studies involving the characterisation of pectic polysaccharide degradation from intact walls plants were harvested at early bud stage and at full bloom. Plants at the appropriate developmental stage were harvested from individual pots and pooled, freeze-dried, and then separated into leaf and stem fractions. Only leaves of early bud stage and stems of full bloom plants harvested in 1988 and 1990 were used in this study. Our purpose was not to study the influence of developmental changes but rather to determine if the degradation characteristics of pectic polysaccharides in highly lignified mature walls (full bloom stems) differed from easily degraded walls (early bud stage leaves). Pooled stems were further subdivided into regions reflecting stem development and only the lower seven to eight nodes and internodes (LN) were used in the degradation studies. For all experiments, isolated leaves and stems were ground to pass a 1.0 mm screen of a cyclone mill (Udy Corp, Fort Collins, CO, USA).

Cell wall isolation

Cell wall (CW) material was isolated as previously described (Hatfield 1992) with minor changes. Briefly, ground plant material was thoroughly mixed for 15 min in cold 5°C phosphate buffer (10 mM NaH_2PO_4 + 50 mM NaCl, pH 7.0, 50 ml g^{-1} dry tissue) in 250 ml centrifuge bottles with the aid of a stir bar and magnetic stirrer. The insoluble residue was pelleted by centrifugation ($1000 \times g$) and the supernatant carefully decanted through a glass fibre filter (GF-4, Fisher Scientific, Pittsburgh, PA, USA). Fresh buffer was added to the bottles, and the residues were stirred and recovered as before. This washing procedure was repeated for cold 50 mM NaCl (50 ml g^{-1} dry tissue, 5°C, twice), followed by acetone (20°C, four times), chloroform/methanol (2:1, once) and finally a single wash of acetone. After the final wash, samples were allowed to air dry in the centrifuge bottle.

Each CW sample was suspended in phosphate buffer containing sodium azide (10 mM KH_2PO_4 + 0.2 g litre^{-1} NaN_3 , pH 7.0, 25 ml g^{-1} CW) and heated in a boiling water bath for 1.5 h. Bottles were cooled to 60–65°C, 10 IU of α -amylase (Sigma A-3403, Sigma Chemical Co, St Louis, MO, USA) were added, and then placed in a waterbath at 55°C for 1.0 h. After the initial incubation, pH was adjusted to 4.75, 10 units of amyloglucosidase (Sigma A-3514) added, and the bottles returned to the waterbath for an additional 1.5 h. It is important to maintain the incubation temperature above 55°C in order to inhibit wall hydrolases present in these enzyme preparations. CW were pelleted at $2500 \times g$ (10 min) following amyloglucosidase treatment. The supernatant was decanted into filtration flask fitted with a glass fibre filter (GF-4 Fisher). Thirty millilitres deionised water was added to each bottle, mixed thoroughly and CW pelleted as before. After four wash cycles the CW were transferred to a freeze-drying flask and lyophilised. The amylase extract and washes were combined, dialysed against water (8–10 litres) for 48 h, frozen and lyophilised. This extract, primarily pectin, was treated as a hot water extract of the CW.

Isolation of pectic polysaccharides

Isolation of bulk samples of pectic polysaccharides for degradation studies

Hot buffer extractable pectic polysaccharides (HW) were isolated as described above in the starch removal step. Starch-free cell walls were extracted with ammonium oxalate, $(\text{NH}_4)_2\text{C}_2\text{O}_4$, to remove Ca^{2+} -bound pectic polysaccharides as previously described (Hatfield 1992). Briefly, CW material was suspended in $(\text{NH}_4)_2\text{C}_2\text{O}_4$ (5 g litre^{-1} , pH 3.5, 150 ml g^{-1}) and continuously stirred in a water bath (70–80°C) for 1 h. After heating, the CW residue was pelleted by centrifu-

gation and the supernatant decanted through a glass fibre filter (GF #4, Fisher). The CW residue was washed once with $(\text{NH}_4)_2\text{C}_2\text{O}_4$ (5 g litre^{-1} , 75 ml) before being resuspended in $(\text{NH}_4)_2\text{C}_2\text{O}_4$ (5 g litre^{-1} , 150 ml g^{-1}) and heated for an additional 1 h (70–80°C). The original extract, the wash, and second extract were combined, dialysed against water for 48 h, and freeze-dried to give the ammonium oxalate (AmOx)-soluble pectic polysaccharides.

Fractionation of cell walls for analysis of pectic polysaccharides

Leaf and stem CW samples, before and after degradation by ruminal microbes, were subjected to CW fractionation following the scheme previously described (Hatfield 1992). Briefly, isolated CW material was treated with hot phosphate buffer (90–100°C) including α -amylase and amyloglucosidase treatment to remove solubilized starch, extracted with $(\text{NH}_4)_2\text{C}_2\text{O}_4$ (5 g litre^{-1} , twice), delignified with sodium chlorite, and extracted with water (70–80°C). Isolated fractions were analysed for total sugars, total uronosyls, and neutral sugar composition.

Pectic polysaccharide degradation experiments

Degradation of isolated fractions

Two pectic polysaccharide fractions isolated from lucerne leaves by chemical extraction (hot buffer solubilisation and ammonium oxalate extraction) were subjected to *in vitro* fermentation by mixed ruminal microflora from a cow fed lucerne. Inoculum for the digestion study was obtained by squeezing rumen digesta through four layers of cheesecloth and the solids rinsed by squeezing with additional McDougall buffer (McDougall 1948) until the original filtrate volume was doubled. The filtrate was continuously gassed with CO_2 during collection. All fermentations were carried out in 50 ml screw-cap polystyrene tubes (Corning) under a CO_2 gas phase in a modified McDougall's buffer (with the addition of 8 mM NH_4Cl , 1.6 mM cysteine HCl, and 1.0 mM Na_2S). In an anaerobic glovebag, pectic substrates were suspended in fermentation buffer at a concentration of 10 mg ml^{-1} and mixed with ruminal inoculant (0.2 ml ml^{-1} of culture medium). After incubation at 39°C for 0, 1, 2, 4, 6, 8, 12 and 24 h, paired samples were removed and supernatants analysed for total sugars, total uronosyls, and neutral sugar composition. All samples were run in duplicate and the entire experiment replicated on a different date. In initial experiments the residues in each tube were also analysed with the same procedures as the supernatants to insure that substrates did not precipitate from solution upon partial degradation. For comparison, other soluble non-starch plant polysaccharides were subjected to *in vitro* fermentation. All substrates were soluble in

the fermentation buffer. At the completion of each incubation time, two tubes were used to measure pH, and a subsample removed for fermentation product analysis. Samples were prepared for analysis by the method of Siegfried *et al* (1984) and analysed by HPLC using a Rainin Dynamax HPLC System with a Knauer differential refractive index detector (Rainin Co, Woburn, MA, USA). Components were separated on a Bio-Rad HPX-87H column (BioRad Life Sciences, Hercules, CA, USA) at 45°C using a solvent system of 7.5 mM H₂SO₄ in 3.4 mM EDTA at a flow rate of 0.7 ml min⁻¹.

Degradation of pectic polysaccharides from intact forage samples

A second set of experiments involved characterisation of pectic polysaccharide degradation from lucerne leaf (early bud stage) and stem (full bloom) samples. Individual samples (200 mg in 20 ml buffer) were inoculated with ruminal fluid (5 ml) and incubated for 0, 2, 4, 6, 8, 12, 24, 36, 48 and 96 h at 39°C. After the appropriate incubation period, absolute ethanol was added to a final ethanol concentration of 80:20 (v/v) and the samples were stored at -20°C until removed for analysis. Undegraded wall materials were pelleted by centrifugation, the supernatants removed, and the pellets freeze-dried. Pellets were resuspended in phosphate buffer and treated with α -amylase and amyloglucosidase as previously described for the removal of residual starch. At the completion of enzyme treatments, samples were brought to 80:20 (v/v) ethanol and insoluble material pelleted by centrifugation, the supernatants removed and the pellets freeze-dried. Sample residues were analysed for total sugars, total uronosyls, and neutral sugar composition.

To evaluate the potential resistance of pectic materials to degradation, larger amounts of leaf and stem materials were fermented to provide sufficient residues for CW extraction. Four fermentation bottles each containing 1.6 g of lucerne stem or leaf material and 20 ml of ruminal-buffer inoculum were incubated at 39°C. After 24 h, contents of all stem or leaf bottles were pooled, brought to 80:20 (v/v) ethanol, centrifuged to pellet insoluble material, and the supernatant removed. Pellets were quickly frozen and freeze-dried. Following starch removal (see section on CW isolation), the insoluble residues were subjected to CW fractionation as described above (Hatfield 1992). The partially degraded wall materials were analysed as described above.

A separate set of degradation experiments using a different cow for inoculum was carried out to compare the degradation patterns of the two isolated pectic polysaccharide fractions with those of leaf and stem samples using the same source of ruminal fluid inoculum. This allows a direct comparison of pectin degradation characteristics between isolated forms and the *in situ* pectic polysaccharides of leaves and stems. Using the inocu-

lum from a different cow did not alter the degradation characteristics, therefore the results reported here are the means of all experiments.

General methods

Isolated polysaccharides were hydrolysed with 2 M trifluoroacetic acid (TFA) at 120°C for 1.5 h. The TFA was removed by evaporation under a stream of dry filtered air. Monosaccharides were analysed by capillary gas chromatography as their alditol acetates using the procedure of Blakeney *et al* (1983). Total sugar (used here to refer to total neutral sugars) was determined by the phenol-sulphuric acid method (Dubois *et al* 1956) using glucose and arabinose as standards. Although there was a small amount of interference from uronosyls no attempt was made to correct for this interference. Total uronosyls were determined using the 3-phenylphenol method (Blumenkrantz and Asboe-Hansen 1973) with galacturonic and glucuronic acids as standards; corrections were made for interference from hexoses in the samples.

Neutral sugar composition of CW residues after degradation was determined using a modified Saeman hydrolysis (1963). CW residues were solubilised in 1.5 ml of 12 M H₂SO₄ followed by dilution to 1.6 M H₂SO₄ and hydrolysis at 100°C for 3 h. Inositol (7.5 mg) was added with mixing to each sample as an internal standard. Samples were cooled and filtered through glass fibre filters before neutralisation with solid BaCO₃. The BaSO₄ precipitate was removed by centrifugation and filtration. Subsamples of hydrolysates (2.5 ml) were passed through ion-exchange columns (for cations Dowex HCR-W2 H⁺ form (Sigma Chemical Co, St Louis, MO, USA, and for anions Supelclean LC-SAX OH⁻ form (Supelco, Bellefonte, PA, USA)) and analysed using the Dionex BioLC carbohydrate system (Dionex Corp, Sunnyvale, CA, USA).

Uronosyl residues were reduced to the corresponding neutral sugars using the method of Taylor and Conrad (1972) as modified by Anderson and Stone (1985). For these experiments NaBH₄ was used in place of the radioactive sodium borotritide. Neutral sugar composition of the CW extracts was determined before and after reduction using the method described for TFA hydrolysis.

All data presented represents the mean of duplicate analyses of replicated samples (2) within completely replicated experiments (2 or 3). Individual experiment replicates (eg soluble pectin degradation characteristics) were performed on separate days. First-order rate constants were calculated according to a simple first-order kinetic equation as described by Mertens and Loften (1980) to model kinetics of fibre digestion. Experiments conducted with the isolated pectic fractions and lucerne

cell wall samples using the same source of ruminal fluid inoculum did not appear to be different from those run on different days with different inocula. Therefore, all data were combined as means across all appropriate experiments.

RESULTS AND DISCUSSION

Degradation and fermentation characteristics of isolated pectic polysaccharides

Characteristics of isolated pectic fractions

Relatively large quantities of pectic materials could be obtained from immature lucerne leaf material using simple extraction procedures. Neither the hot buffer soluble fraction (HW) nor the 5 g litre⁻¹ (NH₄)₂C₂O₄ fraction (AmOx) were pure polysaccharides but rather complex mixtures of pectic polysaccharides similar to those isolated from lucerne stems (Aspinall *et al* 1968; Aspinall and Molloy 1968; Hatfield 1991). Based on the neutral sugar composition and total uronosyls (Fig 1) these fractions would be predominantly rhamnogalacturonans. Nearly all of the neutral sugar components in these fractions are covalently linked to the rhamnogalacturonans (Hatfield 1991). Each fraction contained small amounts of glucose (Glc), xylose (Xyl), and mannose (Man) most likely from the co-solubilisation of xylans, xyloglucans, and mannans. These polysaccharides amounted to less than 50 g kg⁻¹ of the total extracted material.

Degradation characteristics of pectic polysaccharides

It was thought that the complex substitution patterns of these pectic polysaccharides might result in a slowed or restricted degradation pattern leaving fragments that might not be readily utilized by ruminal microbes.

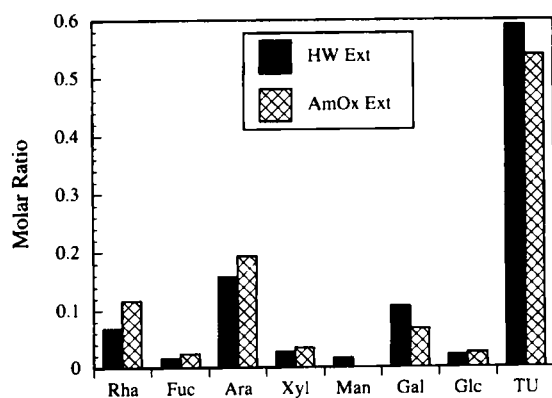


Fig 1. Sugar composition of isolated pectic polysaccharide fractions from lucerne walls. HW Ext, hot buffer soluble pectic polysaccharides; AmOx, ammonium oxalate extract of walls. Rha, rhamnose; Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; TU, total uronosyls.

However, degradation of both isolated pectic fractions by ruminal cultures was rapid having a half-life of approximately four hours and being completely degraded after a 12 h incubation (Fig 2A). Degradation of individual components within these fractions showed some variation (Fig 2A–C). To compare rates, digestion data were analysed using a model for kinetics of fibre digestion described by Mertens and Loften (1980). Degradation rates fitted a simple first-order kinetic equation. Table 1 lists the first-order rate constants for the two isolated lucerne pectic fractions, citrus pectin and cell walls of lucerne leaves and stems. There were no significant differences in the first-order rate constants for uronosyl degradation of the soluble pectic fractions

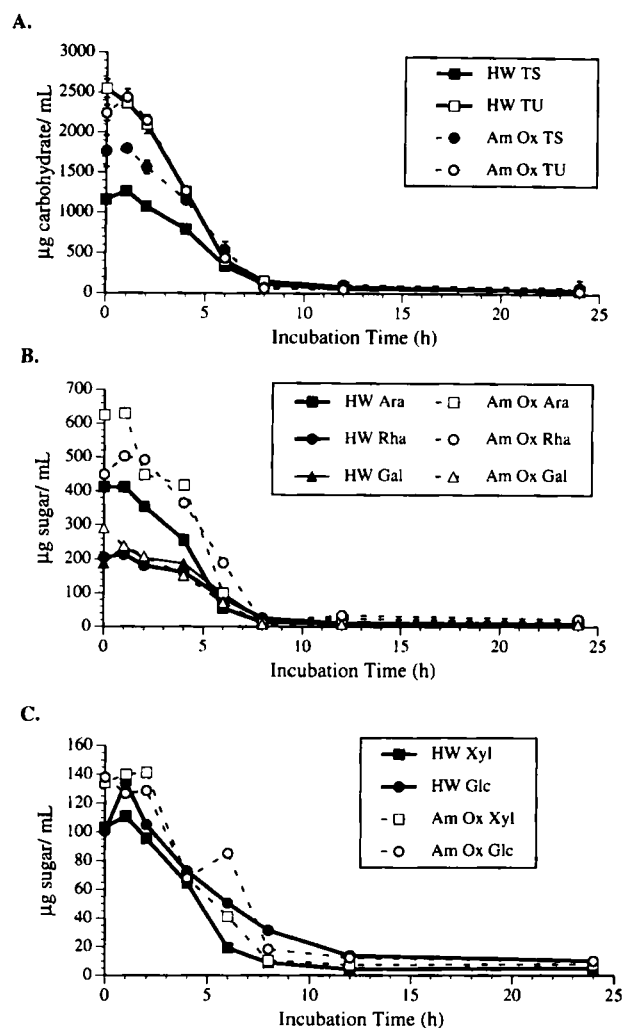


Fig 2. Degradation characteristics of isolated hot buffer soluble (HW) and ammonium oxalate soluble (AmOx) lucerne pectic polysaccharides. (A) Overall degradation pattern of total neutral sugars (TS) and total uronosyls (TU). (B) Patterns of removal of individual neutral sugars from pectic polysaccharides during degradation/fermentation by rumen microbes. Rha, rhamnose; Ara, arabinose; Gal, galactose. (C) Pattern of removal of minor sugars from contaminating polysaccharides co-extracted with the pectic fractions. Xyl, xylose; Glc, glucose.

TABLE 1
Comparison of first-order rate constants (\pm SE) for both isolated pectic polysaccharides and pectic components in intact cell walls ($n = 6$)^a

Sample	First-order rate constant (h^{-1})				
	TS	TU	Ara	Gal	Rha
HW	0.438 ± 0.041	0.339 ± 0.036	0.497 ± 0.075	0.628 ± 0.118	0.423 ± 0.058
AmOX	0.396 ± 0.041	0.500 ± 0.078	0.406 ± 0.061	0.597 ± 0.130	0.377 ± 0.060
CitPec	0.302 ± 0.038	0.487 ± 0.075	ND	ND	ND
CW Lvs	0.138 ± 0.007	0.424 ± 0.066	0.287 ± 0.112	0.255 ± 0.012	0.137 ± 0.088
CW Stm	0.044 ± 0.009	0.251 ± 0.066	0.266 ± 0.049	0.124 ± 0.018	0.096 ± 0.026

^a Degradation rates are based on the linear regression of the logarithmic transformation plots. All plots were the mean of three separate experiments with eight observations per sample. TS, total neutral sugars; TU, total uronosyls; HW, hot buffer extract; AmOX, ammonium oxalate extract; CitPec, commercial citrus pectin; CW Lvs, cell wall of lucerne leaves; CW Stm, cell walls of lucerne stems.

and these were similar to the pectic fraction neutral sugar residues (Ara, Gal and Rha). The complex linkage patterns found in these lucerne polysaccharides did not limit degradation by microbes and no sugar appeared to be resistant to degradation. Each fraction contained a small amount of Glc and Xyl most likely from xylans and xyloglucans that were solubilised during isolation of the pectic materials. Neither Xyl nor Glc of these isolated fractions showed a resistance to degradation.

For comparison citrus pectin (CitPec), polygalacturonic acid (PGA), galacturonic acid (GalA) and arabinogalactan (AraGal) were also degraded by ruminal microbes (Fig 3A). Citrus pectin contained a lower proportion of neutral sugars but had an overall degradation pattern similar to the lucerne fractions (compare Fig 2A with 3A). Arabinogalactan, from larchwood, is a highly soluble polysaccharide fraction that contained a higher Gal composition compared to the lucerne pectic fractions. Despite high solubility, its degradation was limited (Fig 3A and 3B) suggesting linkage patterns that are harder for ruminal microbes to degrade (Fig 3B). Larchwood arabinogalactan is predominantly a Type II arabinogalactan which has a 1 \rightarrow 6 linked Gal backbone with 1 \rightarrow 3 linked substitutions (Stephen 1983; Bacic *et al* 1988), while the linkages found in the lucerne fractions were predominantly 1 \rightarrow 4 linked Gal backbones with Ara substitutions on the 3 or 6 carbon and frequently on both 3 and 6 (Hatfield 1991).

Monomers of GalA were not utilised as rapidly as the polymeric forms of GalA. This may indicate that certain ruminal microbes can more efficiently utilise (or at least transport) oligosaccharides of GalA as opposed to the monomer. Previous work by Gradel and Dehority (1972) indicated that only three strains out of a total of nine pure culture strains in their investigation could readily utilise GalA, but a larger number could utilise pectin substrates either as isolated polysaccharides or

from forage cell walls. The results presented here would support these earlier findings.

Volatile fatty acid (VFA) production and pH changes

As with the degradation, VFA production from the two lucerne pectic fractions was similar. Figure 4 shows acetate and propionate production profiles for the ammonium oxalate extract compared to CitPec,

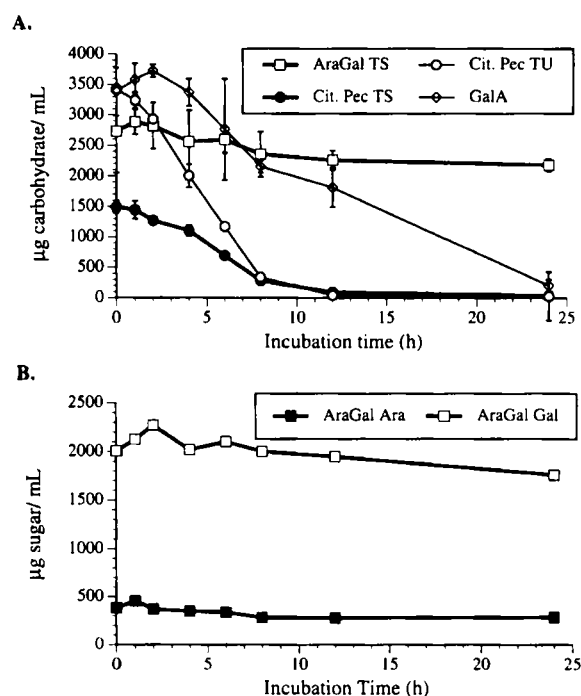


Fig 3. Degradation pattern of other soluble polysaccharides. (A) Total neutral sugar (TS) and total uronosyl (TU) patterns for arabinogalactan (AraGal), citrus pectin (CitPec) and galacturonic acid (GalA). (B) Degradation pattern of arabinose (Ara) and galactose (Gal) in the arabinogalactan (AraGal) polysaccharides. Larchwood arabinogalactan is predominantly a Type II arabinogalactan which has 1 \rightarrow 6 linked galactose backbone with 1 \rightarrow 3 linked substitutions.

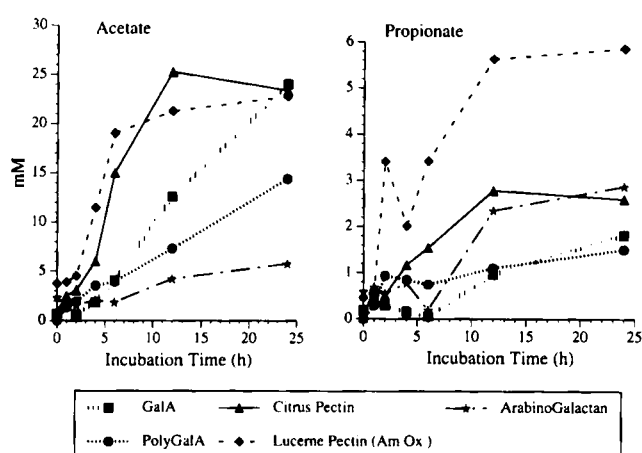


Fig 4. Production of the major volatile fatty acids, acetate and propionate, during the fermentation of soluble substrates; galacturonic acid (GalA), citrus pectin (CitPec), arabinogalactan (AraGal), polygalacturonic acid (PGA) and ammonium oxalate soluble lucerne pectic fraction (AmOx).

AraGal, PGA and GalA. For these carbohydrates acetate and propionate accounted for 90–99 mol% of the total VFA production with butyrate accounting for the remainder. The rate of acetate production was highest for lucerne pectin and citrus pectin with maxima occurring before 6 h of incubation. These results compare well with the degradation characteristics of citrus and lucerne pectic polysaccharides in that between 6 and 8 h there was a decline in the degradation rate (Figs 2 and 3). This suggests that substrate was becoming limited in this system. VFA production paralleled substrate disappearance indicating that these substrates were being utilised rapidly by the ruminal microbes and not merely hydrolysed into individual sugars. Although the total acetate production from GalA was similar to that from the two pectic polysaccharides this level was not achieved until 24 h (versus 12 h for the pectic polysaccharides) reflecting the much slower uptake of GalA described earlier.

The level of total VFA produced by the citrus and lucerne pectic polysaccharides was lower than the amount previously reported for citrus pectin (Marounek *et al* 1985). This is most likely due to our use of a lower concentration of substrate in the incubation buffer (13.3 mg ml⁻¹ versus 4.0 mg ml⁻¹ this study). The average rate of VFA production (0.694 mmol litre⁻¹ g⁻¹ h⁻¹) from citrus pectin for the first 6 h was similar to that of the Marounek *et al* study (0.596 mmol litre⁻¹ g⁻¹ h⁻¹) when comparing similar pH and inocula. Nevertheless the lucerne pectic polysaccharide rates were higher (0.964 mmol litre⁻¹ g⁻¹ h⁻¹). In addition, the acetate/propionate ratio for lucerne pectic polysaccharides was about half that produced by CitPec fermentation (Fig 5). The increased production of propionate from lucerne pectin (Fig 5) probably results from this pectin's high content of rhamnose. Microbial metabolism of this

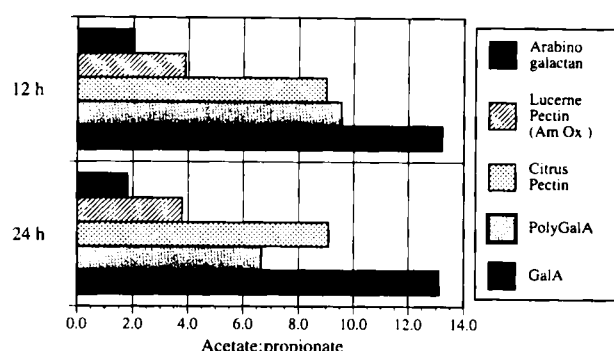


Fig 5. The acetate to propionate ratio after 12 and 24 h of fermentation of soluble substrates; galacturonic acid (GalA), citrus pectin (CitPec), arabinogalactan (AraGal), polygalacturonic acid (PGA) and ammonium oxalate soluble lucerne pectic fraction (AmOx).

deoxyhexose leads to formation of three-carbon intermediates (eg lactaldehyde and 1,2-propanediol) that are readily converted to propionate by many anaerobic bacteria (Turner and Robertson 1979; Czerkawski *et al* 1984; Weimer 1984).

The rapid degradation of the lucerne fractions and CitPec did not result in a pH decline in the rumen buffer system. All substrate pH values were between 6.8 and 7.0 after 24 h of incubation and no lactate was detected at any time. When the pH was monitored every 2 h for the first 8 h of incubation for lucerne pectic polysaccharides, the pH changed slightly ($T_{0h} = 7.1$, $T_{8h} = 6.9$). This was due to the production of predominantly acetate ($pK_a = 4.75$) and the rapid utilisation of GalA ($pK_a \approx 3.75$) from these pectic polysaccharides.

Degradation characteristics of pectic polysaccharides in intact walls

Uronosyl and total neutral sugar degradation characteristics

The general degradation characteristics of 1 mm ground stem and leaf samples incubated with ruminal microbes are shown in Fig 6. Measurement of total neutral sugars (TS) gives a good overall measurement of wall degradation as each sample had been treated with α -amylase and amyloglucosidase to remove residual starch. Based on this parameter it can be concluded that leaf walls were degraded more rapidly and to a greater extent than stem walls. The total neutral sugar (TS) pattern compares well with previous studies with lucerne (Ben-Ghedalia and Miron 1984; Albrecht *et al* 1987; Buxton 1991; Buxton and Brasche 1991; Titgemeyer *et al* 1991; Titgemeyer *et al* 1992). A more important characteristic for this study is the loss of uronosyl residues from wall matrices (Fig 6B) to give an indication of pectic polysaccharide degradation. Although the TS profile gives a good overall assessment of wall degradation, degradation of neutral sugars specifically associated with the

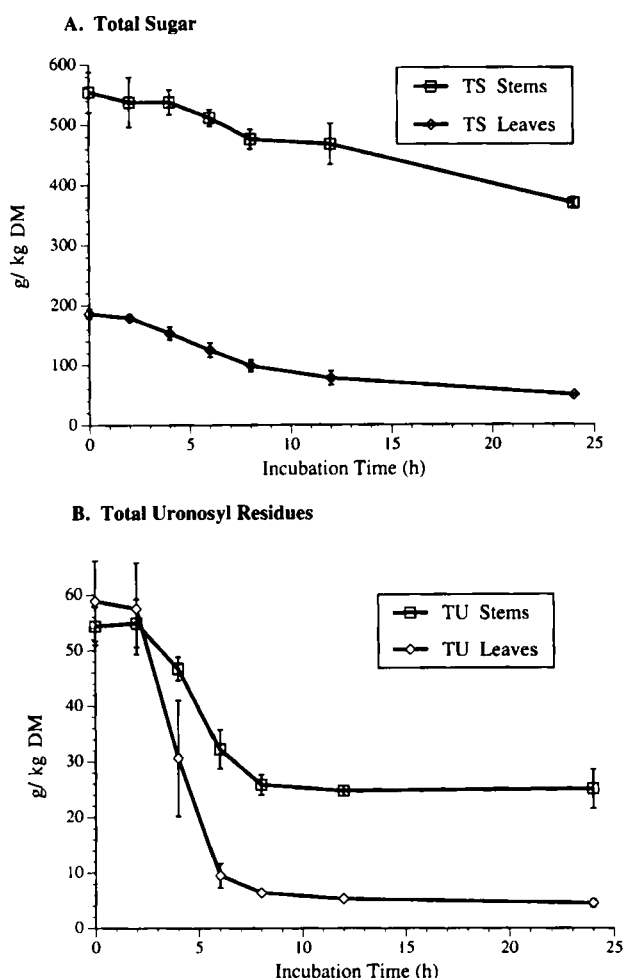


Fig 6. Overall pattern of degradation/fermentation of total cell wall from lucerne stems and leaves by rumen microbes. Undegraded wall material was recovered from rumen microbial fermentation by making the total fermentation buffer 80% (v/v) in ethanol. This treatment immediately stopped microbial activity and precipitated any solubilized yet undegraded wall carbohydrate. (A) Total neutral sugar (TS) profile. (B) Total uronosyl (TU) profile.

pectic polysaccharides are masked by the relatively high amounts of cellulose and xylans which account for 700 g kg^{-1} of leaf walls and nearly 900 g kg^{-1} of stem lower internode walls (see Fig 7A and B).

Three neutral sugars (Ara, Gal, Rha) and galacturonic acid (Gala) are the major constituents of lucerne pectic polysaccharides (Aspinall *et al* 1968; Aspinall and Molloy 1968; Hatfield 1992); therefore, monitoring their changes during fermentation by ruminal microbes provided a more definitive view of how pectic materials were being degraded from wall matrices.

Degradation of uronosyl residues was rapid, reaching a plateau in 8–12 h (Fig 6). A comparison of the first-order constants (Table 1) indicates that uronosyl degradation was faster from leaves than from stems and was essentially the same as those of isolated pectic fractions. Although the uronosyl degradation from stem walls was slower than from the leaves, this first-order rate con-

stant was nearly eight times that of the total neutral sugars (TS). It is interesting to note that the first-order rate constant for Ara was the same for both stems and leaves indicating that wall matrix may not effect degradation of this pectic component. The tissue type and/or maturity differences appeared to have a much greater impact upon the non-pectic polysaccharides in lucerne (Table 1). In addition, extents of uronosyl degradation were quite different between the two tissue types. Uronosyls in stems were approximately 60% degraded while in leaves the level reached 90%. Higher residuals of uronosyls in stems could have been due to resistant rhamnogalacturonans (pectic polysaccharides) or to glucuronosyl residues on xylans that have a slow rate and low extent of degradation, especially in stems (Fig 7; Albrecht *et al* 1987; Buxton and Brasche 1991; Titgemeyer *et al* 1991, 1992).

Degradation characteristics of pectic neutral sugars

The pattern of Ara, Gal and Rha degradation was similar for the two tissues (Fig 7A and B). The graphs show only the first 24 h of degradation although additional time points were taken at 36, 48 and 96 h for these experiments. For the pectic sugars including uronosyls no change was seen in the degradation pattern after 24 h. Only Xyl and Glc showed much change after 24 h. For leaves 90% of the Ara, 72% of Gal and 45% of Rha were degraded in 24 h and for stems 82% Ara, 63% Gal and 33% Rha were degraded. The much lower extent of Rha degradation and the slower rates were not expected based on the degradation pattern of isolated pectic fractions from lucerne (Fig 2 and Table 1). Similar results were obtained by Titgemeyer *et al* (1992) for intact stems. Aspinall and McGrath (1966) identified Rha as a minor substituent on xylans isolated from lucerne. Based on the resistance to degradation of lucerne stem xylans one could assume that a portion of this Rha was from the xylan fraction. However, the proportion of the total Rha associated with the xylan fractions isolated by wall fractionation using KOH was less than the $300\text{--}330 \text{ g kg}^{-1}$ reported by Hatfield (1992) and Titgemeyer *et al* (1992). In addition, the bulk of Rha in these KOH extracts is associated with Ara, Gal and uronosyl rich components that are most likely rhamnogalacturonans that are resistant to ammonium oxalate extraction (Hatfield, unpublished results). Therefore the resistance of Rha to degradation from the wall would most likely be due to specific matrix interactions involving defined regions of rhamnogalacturonan molecules. The chemical nature of such interactions has yet to be defined.

Although Xyl in the isolated lucerne pectic fractions was extensively degraded utilization from intact wall samples was severely limited (Fig 7). These results are similar to those of earlier work demonstrating that isolated polysaccharides are usually completely degraded by ruminal microbes (Dehority *et al* 1962). Structural

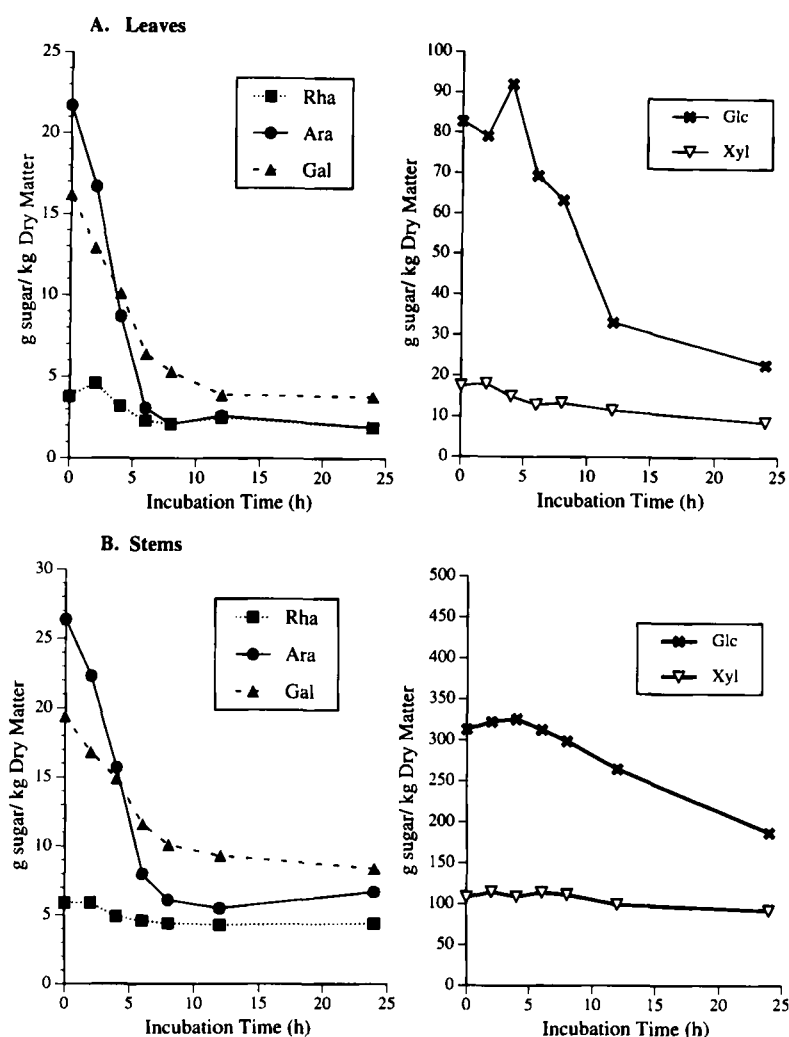


Fig 7. Degradation pattern of individual neutral sugars from stem and leaf cell walls over the first 24 h of degradation/fermentation. Although, additional samples were taken at 36, 48 and 96 h fermentation for pectic polysaccharides little change occurred after 12–24 h of fermentation with rumen microbes. (A) Lucerne leaves. (B) Lucerne stems. Left-hand graphs illustrate degradation patterns for the pectic neutral sugars; rhamnose (Rha), arabinose (Ara) and galactose (Gal). For comparison the non-pectic sugars xylose (Xyl) and glucose (Glc) are shown in the right-hand graphs.

features of the wall (ie lignin, cross-linking interactions) restricting enzyme hydrolysis or limiting accessibility by ruminal microbes or their extracellular enzymes are most likely responsible for limiting individual sugar degradation from wall matrices (Hatfield 1993).

Fractionation of wall samples before and after degradation

Based on the total wall analysis for neutral sugars and uronosyls it was evident that not all of the pectic material was completely degraded from the wall matrix although in leaves this amount appeared to be relatively small. To obtain a better picture of resistant materials, samples of stems and leaves were incubated with rumen fluid for 24 h and the residual materials subjected to wall fractionation. Samples before degradation were also subjected to the same procedure. Figure 8A shows a comparison of the four pectic fractions (HW, AmOx, delignification solubilized material, Lig Ext; and hot

water extraction of walls after delignification, HW Lig) from leaf and stem material before and after degradation by ruminal microbes. Of particular interest was the fraction that was solubilised upon delignification of the walls as it had been previously shown that this treatment released significant amounts of pectic materials (Hatfield 1992). In earlier related work, extraction by hot water and AmOx (Chesson and Monro 1982) or by ammonium oxalate alone (Titgemeyer *et al* 1992) were used as indicators of total pectic materials in wall matrices. For our experiments, as in earlier work, AmOx was in essence completely degraded (>98%) for both leaves and stems while the degradation of other pectic fractions was not as complete (Fig 8A).

As expected stem materials were generally less degradable than leaves. Polysaccharides solubilized by the delignification treatment (Lig Ext) and followed by hot water extraction (HW Lig) did appear to have the largest fraction of resistant pectic material in the stems

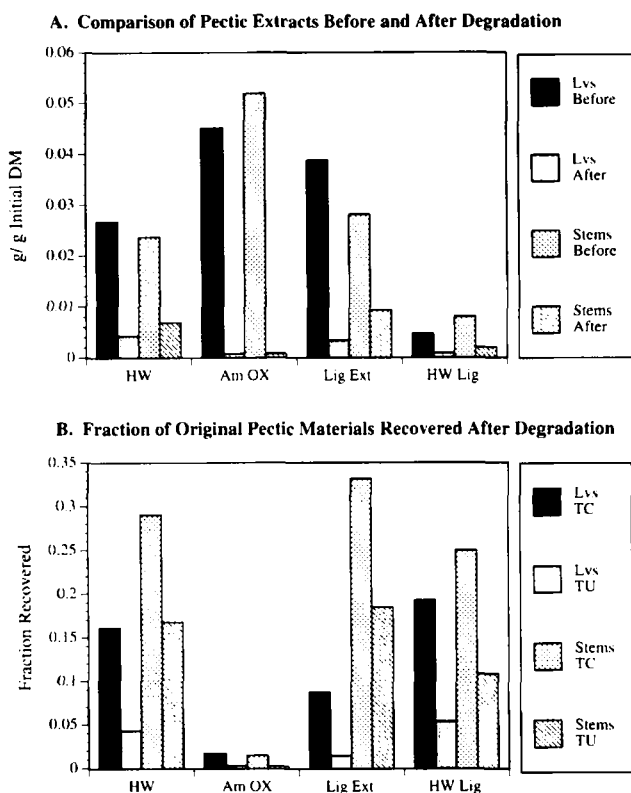


Fig 8. Comparison of total carbohydrate extracted by a pectic polysaccharide isolation scheme before and after degradation/fermentation of leaf and stem material. To determine the impact of wall organisation on pectic material degradation, walls were degraded/fermented for 24 h with rumen microbes. Samples were made 80 : 20 (v/v) in ethanol/water and the pelleted undegraded residues subjected to a pectic polysaccharide isolation protocol; HW, hot buffer soluble fraction; AmOX, ammonium oxalate fraction; Lig Ext, delignification solubilized material; and HW Lig, hot water extract after delignification. (A) Comparison of total extractable material before and after fermentation. (B) Fractional recovery of total carbohydrate (TC = TS + TU) and total uronosyl (TU) in each fractionation step. The fractional recovery is the proportion of TC and TU that was recovered in each extraction step after fermentation of leaf and stems samples for 24 h when compared to the same extraction steps before fermentation.

(Fig 8B). Based on the total carbohydrate (total neutral sugars + total uronosyls = TC) recovered from the Lig Ext and HW Lig treatment, 33% and 25%, respectively, were undegraded. However, neutral sugar compositions of these fractions underwent a significant shift in molar proportions (Fig 9), to increased Rha, Xyl, Glc and Man with decreases in Ara and Gal. Based on the total carbohydrate isolated in the different pectic fractions, before and after degradation, 80 g kg⁻¹ of the leaf and 170 g kg⁻¹ of the stem pectic polysaccharides were left undegraded.

In order to determine the proportion of GalA residues resistant to degradation, uronosyl residues in the pectic fractions after degradation were reduced to their corresponding neutral sugars and analysed. Only the HW, Lig Ext and HW Lig fractions had sufficient

material for reduction. For leaves the HW extract was 58 mol% GalA and 42 mol% GlcA, Lig Ext was 52 mol% GalA and 48 mol% GlcA and in the HW Lig extract 49 mol% was GalA. In the stems these same extracts were 70, 81 and 89 mol% GalA, respectively. Figure 8B shows the fractional recoveries in each pectic fraction after degradation assuming that each extraction treatment was solubilising the same type of polysaccharides before and after partial degradation of the wall matrix. For leaves the undegraded material (80 g kg⁻¹ of the original) was composed of about 220 g kg⁻¹ uronosyls, and for stems (170 g kg⁻¹ of the original) approximately 430 g kg⁻¹ was uronosyls. These results would indicate that for stems a small amount of the pectic uronosyls was resistant to degradation.

It cannot be said with certainty that all of the materials in the pectic fractions after degradation would have been associated with these same fractions before degradation. Nevertheless it is apparent that at least 92 and 83% of the pectic materials are degraded from leaf and stem wall matrices respectively. This is in spite of the observation that approximately 330 g kg⁻¹ of the total pectic material was not amenable to extraction until walls were delignified (Hatfield 1992, and this study, Fig 8). It would seem likely that there are some forms of crosslinking among pectic polysaccharides and other wall components. Whether these wall components are lignin, structural proteins, or polysaccharides (including pectins) remains to be elucidated. It is interesting to note that Rha was not as readily released from wall matrices. This may indicate that Rha is intimately involved in the crosslinking process. Whether it is through some type of direct linkage or through some other substituent (Ara or Gal) attached to it remains to be determined. Fry (1982, 1983) has identified ferulic acid esterified to pectic polysaccharides and has suggested that ferulic acid crosslinking may prevent some pectic polysaccharides from easy extraction (Fry 1986). While the nature of the proposed crosslinks remains to be elucidated, it is clear that different regions of the pectic polysaccharides vary with respect to chemical or biological behaviour.

CONCLUSIONS

These findings indicate that pectic polysaccharides represent a potential energy source that is rapidly degraded from either stems or leaves by ruminal microbes. Lucerne plants with higher total pectic polysaccharides should provide a greater amount of easily utilised energy and may provide additional energy to better utilise the rapidly degraded protein in the rumen. Therefore, selection of forage lines with increased pectic polysaccharides may be of nutritional benefit to the animal.

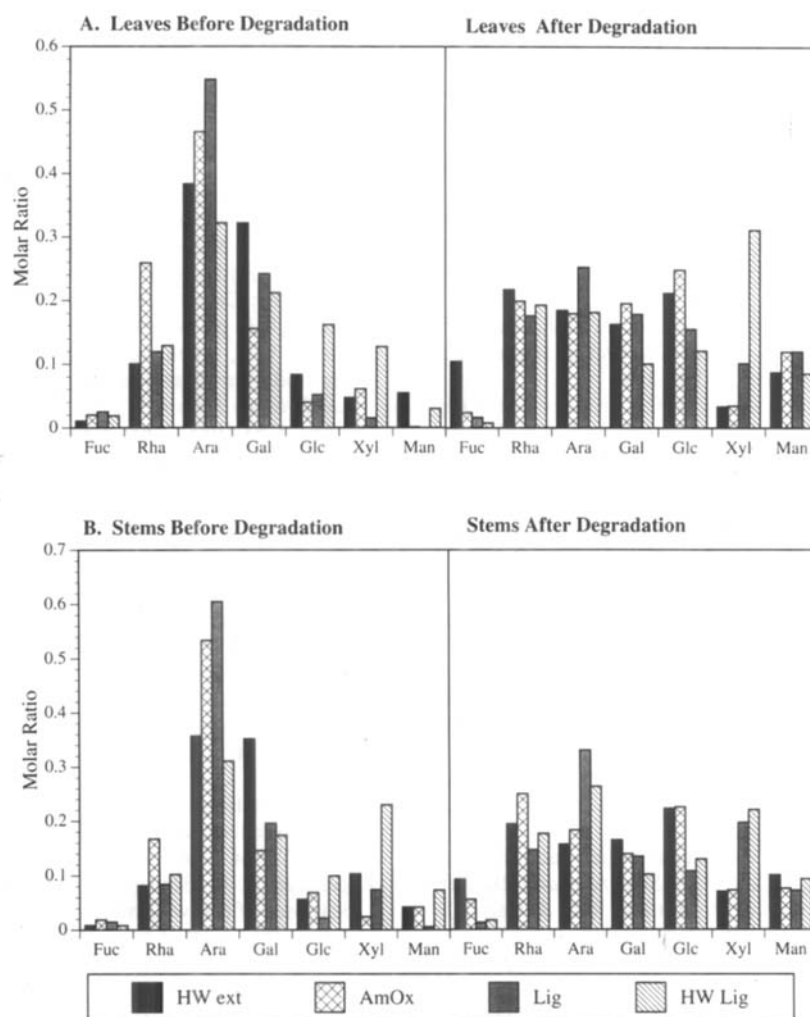


Fig 9. Neutral sugar composition of pectic polysaccharide extracts isolated from walls before and after degradation/fermentation for 24 h by rumen microbes. (A) Leaves. (B) Stems. HW, hot buffer soluble fraction; AmOx, ammonium oxalate fraction, Lig Ext, delignification solubilized material; and HW Lig hot water extract after delignification.

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